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Toxicity study of isolated polypeptide from wool hydrolysate

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ABSTRACT

The cytotoxicity of wool polypeptide has been evaluated by both cell and animal models. Wool was dissolved in sodium hydroxide solution, the pH value of the solution was adjusted to 5.55 and the precipitate was harvested as wool polypeptide. The spray-dried polypeptide was collected as powders and characterized by SEM, FTIR and TG–DSC. The cell culturing results showed that wool polypeptide had no obvious negative effect on cell viability in vitro. Both acute oral toxicity and subacute 30-day oral toxicology studies showed that wool polypeptide had no influence on body weight, feed consumption, blood chemistry, and hematology at any dose levels. There were no treatment related findings on gross or detailed necroscopy, organ weights, organ/body weight ratios and histology. Our study indicated the absence of toxicity in wool polypeptide and supported its safe use as a food ingredient or drug carrier.

1. Introduction

Wool is one of the most abundant natural polymers, which has evolved over thousands of years to insulate and protect sheep. It has been estimated that wool contains about 20 amino acids linked together in ladder-like polypeptide chains (Block et al., 1939). Keratin and polypeptide from wool are renewable, biodegradable, and biocompatible biomaterials that can perform a fundamental structural role in many biological systems. As low cost, standardized alternatives to collagen and fibronectin, keratin and polypeptide have been used in new product developments for the food, textile, pharmaceutical, medicinal, cosmetic, and biotechnology industries (Sun et al., 2012; Lee et al., 2012; Katoh et al., 2004; Tachibana et al., 2005; Xie et al., 2008; Aluigi et al., 2007a; Turowskil and Kaliszan, 1997; Barba et al., 2008; Lv et al., 2008; Belcarz et al., 2009). Particularly, wool keratins consist of several protein species carrying cell adhesion sequences, arginine–glycine–aspartic acid and leucine–aspartic acid–valine. For this reason, these natural biocompatible polymers are attracting a lot of attention in biomedical applications (Humphries et al., 1987; Li et al., 2009; Chu, 2012; Li et al., 2012a,b, 2013). Non-antigenic keratin and polypeptide would have advantages for wound care, tissue reconstruction, cell seeding and diffusion, and drug delivery (Apel et al., 2008; Yamauchi et al., 2003; Banning and Heard, 2002; Heard et al., 2003. As implantable biomaterials, keratin and polypeptide can be absorbed by surrounding tissues to provide structural integrity within the body while maintaining stability under mechanical load (Timmons et al., 2000). They are therefore well suited to the development of medical products with high-value and broad market prospects (Yamauchi et al., 1998).

In order to extract wool keratin and polypeptide, acidic hydrolysis, enzymatic hydrolysis and alkaline hydrolysis are three methods used to hydrolyze wool fibers (Paton et al., 2008; Röper et al., 1984; Shang et al., 2009; Liu and Yu, 2010; Blackburn and Lee, 1956). The microstructure of keratin or polypeptide forms protein homologs of 40–60 kDa by reduction hydrolysis and peptide fractions of 6–8 kDa by alkaline hydrolysis (Cardamone, 2010). Enzymatic hydrolysis has some disadvantages, including high expense, low hydrolysis efficiency and long hydrolysis time (Toran-Diaz et al., 1985). Sodium hydroxide can break the disulfie bond (S—S) and peptide bonds of keratin easily to form water-soluble keratose as the first product of the keratin hydrolysis. The alkaline nucleophilic treatment provides a pathway for keratin modification through the degradation of cysteine leading to thio-ether linkages, and has been extensively used in the hydrolysis of wool because of its high efficiency and low cost (Hikima and Nonomura, 2008).

Although wool keratin and polypeptide have been widely studied in tissue engineering, the evaluations were focused on full keratin and polypeptide without further separations. However, it was demonstrated that different polypeptides or amino acids had

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diverse physical and chemical characteristics (Chen et al., 2009). The isoelectric point is the pH value at which a particular molecule or surface carries no net electrical charge. Isoelectric precipitation is a process in which proteins or amino acids are precipitated at a pH value of solution close to their isoelectric points. This characteristic property can be applied in the crystallization of protein, amino acid or peptide (Gigliotti et al., 2008). The current study isolated a specific part of polypeptide at pH 5.55 from the alkaline hydrolyzed wool solution and examined its toxicity using cell and animal models. It is expected to be a potential natural base material for drug delivery, wound healing and other medical applications.

2. Materials and methods

2.1. Fabrication and characterization of polypeptide

Wool fiber (100 g, domestic fleece of fine grade, Shandong, China) was immersed in 1.0 l 0.5 N sodium hydroxide (Acros, Belgium) solution, then incubated at 80–90 °C in a water bath for 2 h to hydrolyze the wool completely. Afterwards, the pH value of the hydrolysate was adjusted to 5.55 by adding 0.1 N hydrochloric acid. After the polypeptide was precipitated overnight it was collected and named as PP5.55. Then PP5.55 was re-suspended in deionized water and harvested by centrifugation at 5000 rpm for 10 min. The washing procedure was repeated five times to remove any unreacted ions. Finally, the polypeptide suspension was spray-dried to obtain PP5.55 powder. The inlet temperature of the spray-drier was set at 200 °C. By adjusting the feed rate of the polypeptide suspension (about 6 ml/min), the outlet temperature was controlled at 110–115 °C. The spraying air flow was 380–400 normlitre/h. The gas flow of the Aspirator was 32–35 m^3/h. The yield was 30 ± 1.5%. The product was composed of polypeptide fragments of 6–8 kDa with a protein content of 80.0 ± 0.2%.

For SEM, PP5.55 powders were directly adhered onto an aluminum stub with a thin self-adherent carbon film and then coated with a thin layer of gold. The shape and morphology of the samples were observed under a scanning electron microscope (SEM, JEOL, JSM-6490, Japan).

Fourier transform infrared (FTIR) spectra for the samples were obtained on an FTIR Perkin Elmer 1720 (Perkin Elmer, USA) in the transmission mode at wavenumbers ranging from 4000 to 450 cm^{-1}. Potassium bromide (KBr) pellets were prepared by gently mixing the sample powder with KBr.

The thermal properties of the samples were measured by thermogravimetry–differential scanning calorimetry (TG–DSC, Netzsch STA 449C, Burlington, Germany) at a heating rate of 10 °C/min over a temperature range of 30–400 °C.

2.2. Cell toxicity

2.2.1. Cell culture

Human foreskin fibroblast cell line (HFF-1, American Type Culture Collection, USA) was cultured in 25 cm^2 culture flasks (SPL, Korea) with Dulbecco's modified medium (DMEM, Sigma, USA) at 37 °C and 5% CO_2. DMEM was supplemented with 10% fetal bovine serum (FBS, Caisson, USA) and 1% penicillin–streptomycin solution (Caisson, USA). Cells were harvested by adding 0.25% trypsin with EDTA Tetrasodium (Caisson, USA). After the cells were detached, the trypsin was neutralized by adding DMEM media with 10% FBS. Cell suspension was centrifuged and re-suspended with fresh medium and sub-cultured for the particular experiments.

2.2.2. Preparation of polypeptide suspensions

PP5.55 powders were dispersed into phosphate buffered saline (PBS), treated by ultrasonics for 30 min. The polypeptide/PBS suspensions with different concentrations (0.001; 0.01; 0.1; 1.0 and 5.0 mg/ml) were added to the medium at the same volume ratio (1:9) before each experiment. The final concentrations of PP5.55 in the medium were 0.0001, 0.001, 0.01, 0.1 and 0.5 mg/ml, respectively.

2.2.3. Morphological observation

HFF-1 cells were seeded into six-well plates (Nunclon™ Surface, Nunc, Denmark) at the density of $2 \times 10^4/cm^2$. After the cells were incubated for 24 h, they were stained using 1.0 mg/l fluorescein diacetate (FDA, International Laboratory, USA). The morphological status of the fibroblasts was observed by a fluorescent microscope (ECLIPSE 80i, Nikon, Japan) using 4× magnification.

2.2.4. Cell number counting

HFF-1 cells were seeded into 24-well plates (Nunclon™ Surface, Nunc, Denmark) at a density of $1 \times 10^4/cm^2$. After incubation with polypeptide for 24 h, the cells were detached, centrifuged and re-suspended into a medium containing 0.4% trypan blue (Invitrogen, USA). The numbers of cells were counted using a haemocytometer (Precicolor HBG, Germany). The cell numbers were normalized by the controls and expressed as the percentage of cell viability.

2.2.5. Alamar Blue assay

After the cells were seeded into 24-well plates at a density of $1 \times 10^4/cm^2$ and cultured for 24 h, the medium was removed from each well. Alamar Blue solution (Invitrogen, USA) was added to each well and incubated for 4 h. The absorbency of the medium was obtained via a Micro-plate reader (infinite F200, TECAN) at a wavelength of 570 nm. The absorbency values were normalized by the controls and expressed as the percentage of cell viability.

2.3. Animal model

All animal experiments were performed in compliance with the local ethics committee. All mice and rats were provided by the Experimental Animal Center of Xi'an Jiaotong University in Xi'an, China. The animals were housed in polypropylene cages with stainless steel grill tops and provided with bedding of clean paddy husk. The animals were acclimatized to laboratory conditions for 1 week prior to the experiments. The temperature in the animal room was maintained at 25 ± 2 °C with a relative humidity of 40–60% and the illumination cycle set to 12 h light and 12 h dark. The mice and rats were fed a standard laboratory diet and tap water *ad libitum* for a week before the experiments. All experiments were performed in accordance with the Animal Experimentation Committee Regulation (China).

2.3.1. Acute oral toxicity study

Fifty male and 50 female ICR mice (aged 6–8 weeks, weighing 18–22 g) were randomly divided into five groups: four treatment groups and one control group, 10 male and 10 female mice for each group. PP5.55 powder was mixed in distilled water and administered to the mice via oral gavage at doses of 50, 500, 1000 and 5000 mg/kg body weight per day, respectively. After gavage, the mice were observed for 15 days to observe their symptoms and mortality. Negative (by distilled water) controls were recorded at the same time.

2.3.2. Subacute oral toxicology study

Healthy Sprague–Dawley postweaning rats (weighing 145–155 g) were randomly divided into four groups: three treatment groups and one control group, 10 males and 10 females for each group. PP5.55 powder was mixed in distilled water to prepare different test substances (1.0, 10 and 100 mg/kg). Distilled water was given as a negative control. After oral gavage, the rats were observed for 30 days and their symptoms, mortality and body weight were recorded. Afterwards, the surviving animals were anaesthetized by i.p. injection of ketamine hydrochloride (350 mg/kg; Inoketam100; Virbac; Milan, Italy) and bled to death through the abdominal aorta.

2.3.2.1. Coefficients of organs. The tissues and organs, including liver, left and right kidneys, heart, lung, spleen, brain, uterus and ovary, left and right testicle, were excised and weighed accurately. The ratios of organs to body weight were calculated (wet weight).

2.3.2.2. Hematology and clinical biochemical parameters. Blood samples, collected via the ocular vein (about 0.8–1.0 ml each rat), were centrifuged twice at 3000 rpm for 10 min in order to separate the serum. In the present study, the liver function was evaluated with serum levels of alanine-aminotransferase (ALT), aspartate-aminotransferase (AST). Nephrotoxicity was determined by blood urea nitrogen (BUN) and creatinine (CRE).

2.3.2.3. Histological analysis. Heart, liver, spleen, lungs, kidneys, brain, testis and ovaries were embedded in paraffin and sectioned (5 μm in thickness). Sections were de-paraffinized, rehydrated and stained with hematoxylin–eosin, following standard techniques for histological analyses by light microscopy. Histopathological examination by light microscopy was carried out in a blinded way with respect to treatment.

2.4. Statistical analysis

All data were summarized in a tabular form and expressed as a mean ± standard error and significant differences between the control and experimental groups were calculated using the Student's *t*-test, accepting *p*-values lower than 0.05 as significant.

3. Results

3.1. Morphology of PP5.55 powders

Scanning electron microscopy (SEM) identified the morphology of the surface layer. Fig. 1 shows the SEM of the PP5.55 powders which were collected by spray-drying. Their sizes were about 1.0–10 μm. During the spray-drying process, the small drops of